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Original Article

A novel combined method for cost–benefit production of DNA ladders

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Abstract

Background: Molecular deoxyribonucleic acid markers are one of the most important tools in molecular biology labs. The size of DNA molecule is determined by comparing them with known bands of markers during gel electrophoresis. In this study, we have suggested an efficient strategy to produce molecular weight markers in an industrial scale.

Materials and Methods: A combination of two previously known methods, restriction enzyme digestion and polymerase chain reaction (PCR), was used. The enzymatic digestion process was based on designing and constructing plasmids which equaled in size with the bands of ladder and produce the DNA fragment by plasmid linearization through digestion. In the PCR method, the DNA fragments with length 102 bp lesser than the related bands in DNA ladder are amplified by PCR and cloned in pTZ57T/A cloning vector. Then, PCRs with forward and reverse 100-bp primers on the resulting plasmids amplify the ladder fragments. F100 and R100 primers bind to the backbone of pTZ57R (without insert) and amplify a 100-bp PCR product. PCR on the plasmid with insert amplifies DNA fragment with 102+ insert length bp size.

Results: Upon application of this strategy, 2000-10,000 bp DNA fragments were produced by enzymatic digestion of plasmids of the same size. Moreover, 100-1500 bp fragments were produced during PCR using only a set of forward and reverse (100 bp) primers.

Conclusion: The highest advantage of this cost–benefit approach is to produce different types of molecular weight markers by using an effective and short protocol.

Key Words: DNA ladder, DNA marker, molecular weight

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INTRODUCTION

Concurrent with the growth of molecular genetics techniques, such as using restriction endonuclease enzymes and Polymerase Chain Reaction (PCR), DNA markers are used as essential tools for estimation of DNA size fragments in an electrophoretic field. Standard DNA markers are DNA fragments with

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various sizes. One of the simple methods for preparation of DNA markers is enzymatic digestion of identified map-restricted phages or plasmids. The size of the fragments resulting in this approach is dependent on the composition of genomic base, kind of applied restriction enzyme, and reaction condition. The most used phages and plasmids for this purpose are lambda phage and ΦX174, and pUC and PBR322, respectively (Fermentas, Vilnius, Lithuania). Among the digest markers, enzymatic restriction of lambda phage with *Hind* III is the most popular method for DNA marker production. Another approach is partial digestion of genomic cell DNA which contains many tandem repeat units.[1] The produced nucleic acid fragments are called DNA markers. Despite its simplicity, there are some problems in practice for production of DNA markers in this way, including lack of increasable standard pattern, variety in fragment concentration due to size difference, and complexity of the produced fragments. To circumvent these obstacles, new nucleic acids markers, called DNA ladders, have been developed which have standard pattern fragments.[2] The popular method for DNA ladder production is PCR. In this approach, fragments can be designed in a defined size so as to provide a standard pattern, but some problems such as cost and complexity of production still remain. Complexity of production is due to multiple set primer design/synthesis and applying several PCR apparatus programs. Therefore, many planes have been designed to overcome the mentioned hindrances, including multiplex PCR[3,4] and PCR-synthesized markers (PSM).[5] Another suitable approach is digestion of engineered plasmids containing particular restriction sites for one or more restriction enzyme (s) with standard size units. These plasmids can be propagated in bacterial systems and subsequently digested by the respective enzymes.[6,7]

Nowadays, DNA markers are made in a large scale mainly by two methods. As described above, the simplest way is enzymatic digestion of plasmids or phages by restriction endonucleases. Despite its simplicity and cheapness, this approach produces a non-uniform pattern of fragments, which restricts its application in industrial production. An ideal DNA marker has defined bands with the same concentration. To achieve this goal, PCR can produce efficient amount of DNA fragments in a short time for more simplicity in production process. Multiplex PCR method can be employed, but both these approaches suffer from the disadvantage of using several sets of primers which leads to increase in cost. So, these approaches cannot achieve industrial capability of production. To bypass this problem, the PSM was developed to produce various DNA fragments only by one pair of primer. The range of produced fragments by this method is 100-1000 bp. Another promising approach for industrial production and preparation of engineered plasmids contains the desired and unique restriction sites for producing DNA fragments. In this manner, all produced fragments are considered as one band in a DNA ladder, in contrast to previous plasmids in which some digested fragment must be eliminated from the final DNA ladder. In this study, we suggest a combined method for DNA ladder production that is based on PCR and digestion, therefore large and small fragments are produced by digestion on designed plasmids and PSM method, respectively.

MATERIALS AND METHODS

Plasmids and cloning kits

Restriction enzymes, T/A cloning kit, MiniPrep plasmid kit, and gel extraction kit were purchased from Fermentas. The basic plasmids which were used for this study included pTZ57T/A vector (Fermentas), pIRES-EGFP (Clontech Laboratories Inc, Mountain View, California, USA), pHH (plasmid contains human beta globin + hygromycine genes in pBGGT backbone), and pUSHH (plasmid contains 2 kbp upstream of human beta globin + beta globin + hygromycine genes in pBGGT backbone). Top10 F` (Invitrogen, Carlsbad, California, USA) was used as the bacterial strain for all steps. All PCRs used Taq DNA polymerase (Cinagen, Tehran, Iran).

Construction of 100-bp DNA ladder

At first, common forward (FC100) and reverse primers (RC100) were designed so that PCR with these primers on pTZ57R amplified a 100-bp DNA fragment. If an insert is T/A cloned in pTZ57T/A vector, size of the resulting PCR product with FC100 and RC100 is 102 bp more than that of the insert. For construction of other fragments (200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, and 1500 bp), the desired size PCR products were T/A cloned into the pTZ57T/A vector. For example, in the case of 700-bp fragment, a 598-bp PCR product was used for T/A cloning. PCR on the resulting plasmids by FC100 and RC100 primers produced the desired fragments of size from 100 to 1500 bp. The 2000-bp fragment was produced by PCR on pTZ57R backbone which covered ampicillin-resistant gene (amp^r) and origin of replication. A restriction site *EcoR*I was added to both terminal primers for circulation of PCR product after digestion with the respective restriction enzyme. The 3000-bp fragment was produced by T/A cloning of a 112-bp PCR product into the pTZ57T/A vector. The resulting plasmids, 2000, 2500, and 3000 plasmids, were propagated in Top10 F` *Escherichia coli* and subsequently digested with *EcoR*I (2000, 3000) and *Hind*III (2500) enzymes.

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Construction of 1-kb DNA ladder

The 250- and 750-bp fragments were produced as PSM method. In this manner, 148 and 648 PCR products were T/A cloned into the pTZ100 separately and then PCR was performed with FC100 and RC100 primers for each fragment. The 2500-bp fragment was produced by PCR on pTZ57R backbone which covers *amp*^{r} gene and the origin of replication (2494-bp fragment). A *Hind*III restriction site was added to both terminal primers for re-circulation of PCR product after digestion with the respective restriction enzyme (*Hind*III). The 3500-, 4000-, and 5000-bp fragments were constructed by T/A cloning of 612-, 1112-, and 2112-bp PCR products in pTZ57T/A vector. Then, the generated plasmids were subjected to digestion by *EcoR*V restriction enzymes to produce the desired fragments. In the same way, 720-, 1416-, and 1170-bp PCR products were cloned into pIRES-EGFP, pHH, and pUSHH, respectively, to produce 6000-, 8000-, and 10,000-bp plasmids. Similarly, *EcoR*I and *Kpn*I were applied for digestion of pHH, pUSHH, and pIRES-EGFP plasmids, respectively.

Purification of produced fragments

PCR-produced fragments were run under electrophoresis on 1% agarose gel and continually gel extracted by gel extraction kit as described in the manual (Fermentas) and finally stored at −20°C for final formulation. Fragments which were generated by enzymatic digestion were subjected only to cleanup and the collected fractions were stored at −20°C. At the end, all purified fragments were added to DNA loading buffer containing 10% glycerol, 10 mM Ethylenediaminetetraacetic acid (EDTA), 10 mM Tris-HCl, and 0.005 g Bromophenol blue (pH 7.6) at defined concentrations.

RESULTS

In the enzymatic process, the five plasmids were successfully constructed based on pTZ57R which could be propagated in Top10 F` *E. coli* strain, because of its origin of replication and *amp*$^r$ gene. The other generated plasmids, pGFPIRES, pHH, and pUSHH, also had the same properties and could be amplified by the same system. All propagated plasmids were extracted from Top10 F` *E. coli* strain and subjected to enzymatic digestion by cheap restriction enzymes such as *EcoR*I, *EcoR*V, and *Kpn*I to produce the desired size ladders [Figure 1a and b].

In the PSM method, p100 to p1500 plasmids were generated by T/A cloning of size-variable PCR products into the pTZ57T/A vector. Then, the resulting plasmids were used as templates in PCRs with only one common set primer (FC100 and RC100 primers).[8] Finally the purified fragments were added to the loading buffer at different concentrations as listed in Table 1 to produce 100-bp and 1-kb DNA ladders.

DISCUSSION

The 100-bp and 1-kb DNA ladders are used frequently in molecular biology as well as gene manipulation techniques. The common generating approach for production of DNA markers is enzymatic restriction of phage and plasmids using restriction endonucleases (Fermentas). However, generation of a precise and ordered DNA ladder is not achieved by them. So, for attaining this goal, two methods were applied: (1) engineered plasmids which contained the provided restriction sites in a defined location overall on the plasmid[6,9] and (2) applying PCR-based methods for generating each fragment.[3,4] Enzymatic digestion, despite having economic advantages, has some problems in practice. For example, according to the proposed method by Hartley,[10] appropriate repeat units were cloned into the basic plasmids. But the plasmid backbone at these engineered plasmids was not considered as a DNA fragment. Therefore, after digestion of these plasmids by the respective restriction enzymes, the backbone fragment interference by other fragments must be eliminated

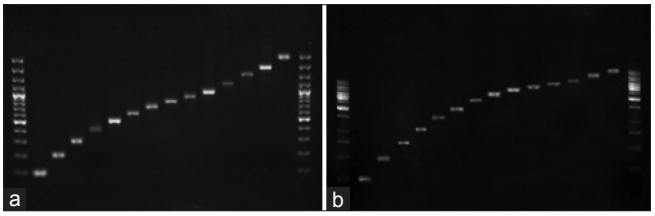

Figure 1: Gel electrophoresis of purified fragments on 1% agarose gel: (a) 100-bp and (b) 1-kb DNA ladders. The standard DNA ladders were purchased from Fermentas Company

Table 1: Concentration of each fragment in loading buffer; 100-bp DNA ladder (upper row), and 1-kb DNA ladder (bottom row)

100-bp DNA ladder													
Fragment (bp)	100	200	300	400	500	600	700	800	1000	1200	1500	2000	3000
Conc. (ng/μl)	6	6	6	6	16	5.4	5.4	5.4	16	5.6	5.6	5.6	5.6
1-kb DNA ladder													
Fragment (bp)	250	500	750	1000	1500	2000	2500	3000	4000	5000	6000	8000	10,000
Conc. (ng/μl)	6	6	6	16	6	6	6	16	6	6	16	6	6

DNA: Deoxyribonucleic acid

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by additional purification steps.[6,11] Generation of fragments by PCR or multiplex PCR[3] method needs several primers leading to complication of production process. PSM[5] method was developed by Chang and his colleagues to decrease the required primers. PCR-based method shows good results for short DNA fragments because when the size of fragments increased, Taq DNA polymerase, the most used DNA polymerase, was not applied due to its low processivity. Therefore, long PCR method with higher cost must be used. In this study, we used a combined method which was economic and simple. The engineered plasmids had the exact size of DNA fragment, so they needed only digestion with respective enzymes. After digestion, a simple cleanup method was used for elimination of reagents such as restriction enzyme or buffer. Since there is no possibility for designing small fragment as a plasmid contains the origin of replication and an antibiotic resistance gene, the best method for production of fragments less than 1500 bp is PCR. In this regard, we designed a plasmid library that contains the desired fragments as templates which could propagate 100-1500 bp fragments by only one pair of primers. This plasmid library overcomes the common disadvantage of PCR, e.g., all reagents in PCR are the same except the DNA template. All the produced fragments were added to the loading buffer containing EDTA to inhibit nuclease activity in the solutions. Here, we used agarose gel electrophoresis and gel extraction in the purification step. In industrial scale, SP Sepharose gel filtration can be substituted to decrease the cost and provide high-purity DNA fragments. In our method, either in PSM or in enzymatic reaction approach, each fragment was generated separately, therefore it was possible to formulate fragments at the desired concentration which increased the quality of the final product and remained consistent.

In conclusion, the novel combined method, including PCR and digestion, is an amazing tool for simple and low-cost production of 100-bp and 1-kb DNA ladders. DNA fragments picked up for the PSM method are short and take less time and cost. In enzymatic digestion, the constructed plasmids could be digested only with low-cost restriction enzyme. In addition, since fragments are produced individually, purification steps can be more done easily than the other methods.

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